TITLE OF THE INVENTION

STABILIZATION OF TRIPLEXES BY WATER STRUCTURE-MAKING SUBSTANCES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 60/034,592 filed January 2, 1997.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The United States Government may have certain rights in this invention by virtue of NIH Grant GM42936 and NIH Biophysics Training Grant GM08309.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to methods for stabilizing nucleic acid triplexes.

2. Description of Related Art

Oligonucleotide third strands can bind to double-stranded nucleic acids to form triple-stranded helices (triplexes) in a sequence specific manner. The third strand binding code (a complementarity principle) dictates the sequence specificity for binding third strands in the major groove of double-stranded nucleic acids to form a triple-stranded helix or triplex. The code provides the specificity of third-strand binding for design of gene-based therapeutic agents that bind specifically to target nucleic acid sequences with little or no non-specific binding to non-target sequences. The third strand binding code, as well as various utilities for triplexes, are described in United States Patents 5,422,251 and 5,693,471 to Fresco, which also shows ionic conditions such as the

presence of Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, Li^{+}, K^{+} or tetramethylammonium cations suitable for triplex formation.

SUMMARY OF THE INVENTION

The present invention relates to methods for enhancing the stability of a triplex formed from one or more nucleic acid strands in a solution, said method comprising adding to the solution, either before or after formation of the triplex, an effective amount of either of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine.

The present invention further relates to a method for forming a triplex from one or more nucleic acid strands, said method comprising adding to a solution, in any order, the strand(s) and an effective amount of one of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine;

and allowing said triplex to form.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, it has been discovered that water structure-making substances can stabilize triplexes in solution. By water structure-making substance is meant a substance which, when dissolved in water, will yield ions or other structures which interact with water more strongly than bulk water molecules with each other.

The water structure-making substances include organic cations, cationic lipids, organic anions, inorganic anions,

and water-miscible organic solvents. Preferred organic cations include alkylammonium (e.g., methylammonium, dimethylammonium, trimethylammonium, tetramethylammonium, and tetraethylammonium, triethylammonium, and their derivatives). Preferred cationic lipids include cetyltrimethylammonium, tridodecylmethylammonium, and 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanammonium and their derivatives. Preferred organic anions include acetate and its derivatives. Preferred inorganic anions include phosphate, sulfate, *etc.*, termed kosmotropes below. Preferred organic solvents include DMSO and alcohols, most preferably methanol, ethanol, 2-propanol, isopropanol and their derivatives.

The oligonucleotide third strand is a synthetic or natural oligonucleotide capable of binding with specificity to a predetermined target region of a double-stranded native nucleic acid molecule to form a triple-stranded structure. The third strand may bind solely to one strand of the native nucleic acid molecule, or may bind to both strands at different points along its length. The third strand need not be perfectly complementary to the duplex, but may be substantially complementary. In general, by substantially complementary is meant that one mismatch is tolerable in about every 10 base pairs.

The oligonucleotide may have a native phosphodiester backbone or may be comprised of other backbone chemical groups or mixtures of chemical groups which do not prevent a triple-stranded helix from forming. These alternative chemical groups include phosphorothioates, methylphosphonates, peptide nucleic acids (PNAs), and others known to those skilled in the art. Preferably, the oligonucleotide backbone is phosphodiester.

The oligonucleotide may also comprise one or more modified sugars, which would be known to those skilled in the art. As an example, such a sugar can be an α-

enantiomer.

The third strand may also incorporate one or more unnatural (for nucleic acids) heterocycle base substitutes if such is necessary or desirable to improve third strand binding. Examples of such unnatural heterocycle design and the heterocycles so designed are found in the co-pending U.S. application of Fresco, *et al.* entitled "Residues for Binding Third Strands to Complementary Nucleic Acid Duplexes of any Base-Pair Sequence", S.N. 08/473,888 filed June 7, 1995, the contents of which are incorporated herein by reference.

The third strand may also contain one or more of a variety of other substituents which can strengthen third strand binding to the target duplex. These include intercalators, crosslinkers, peptides, oligosaccharides, and their analogs and/or derivatives

While the triplex is preferably formed from three discrete strands (two strands which form the duplex target via Watson-Crick binding, and a third strand probe), the present invention also encompasses stabilization of triplexes formed from less than three discrete strands. For example, the triplex may be formed from a single stranded target, and a probe strand that has a sequence complementary to the target strand to form the target duplex, as well as a sequence at a different position which will bind to the formed duplex as if it were a third strand. Further, the triplex may be formed from a target duplex which comprises a single strand which hybridizes to itself via a hairpin turn, and a third strand probe. The triplex may also be formed from a single strand which forms a triplex by virtue of two hairpin turns.

The order of addition of the components of the invention is not critical. For example, the water structure making substance(s) may be added to a solution which already contains the triplex to be stabilized, or may be added along with one or more strands. Moreover, the water structure-making substance may be covalently linked

to the third strand in a manner which would be readily apparent to one of ordinary skill.

The term "solution" as used herein is intended to include both *in vitro* and *in vivo* environments. When dealing with *in vivo* solutions (*i.e.*, in a cell), it will be recognized that toxicity concerns will affect the nature and concentration of water structure making substances that can be employed. In general, cationic lipids will be preferred when dealing with *in vivo* solutions, and may be formulated with the third strand for cellular uptake in a manner known to those of ordinary skill.

The optimum concentration of water structure-making substance to be added may readily be determined by one of ordinary skill. Appropriate concentrations for many substances are set forth in the examples and tables *infra*.

While not wishing to be bound by any particular theory of how the present invention works, it is known that when a salt is dissolved in water, different anions and cations are observed to decrease, increase or have little effect on the volume of the solution. These alternative effects have been explained in terms of the interaction of the anion or cation with water molecules according to what is often called the multilayer hydration model. Briefly, this model of ion-water interaction divides the volume of an ion in solution, V_{ion}, into four components:

$$V_{ion} = V_{cryst} + V_{elect} + V_{disord} + V_{caged}$$

where: V_{cryst} is the volume of the ion based on its crystal radius; V_{elect} is the electrostriction volume (stronger ion-H_2O interaction decreases volume); V_{disord} is the disordered or void-space volume (weaker ion-H_2O interaction increases volume); and V_{caged} is the caged or structured volume (that occurs when a hydrophobic ion (organic cation) interacts with H_2O molecules, which decreases volume).

Although these ion volume factors are interdependent, the observed solution volume changes on addition of ions is

readily explained by this descriptive model. Using this model, ions can be divided into three classes: 1) electrostrictive "structure-making" ions when V_{elect} is dominant; 2) disordered "structure-breaking" ions when V_{disord} is dominant; and 3) hydrophobic "structure-making" ions when V_{caged} is dominant.

The volumes, V_{elect}, V_{disord} and V_{caged} have been calculated for a number of anions and cations (see Horne, R.A. (Ed.)(1972) *Water and Aqueous Solutions*, Wiley-Interscience, NY). As these volumes are additive, predictions of the solution volume effect of a particular salt can be made. The structure-making or structure-breaking tendency of anions based upon this model follows the rank order of the Hofmeister series, which is the relative tendency of anions to stabilize and solubilize proteins. A partial rank order is:

(destabilize proteins) ClO_4^- < Cl^- < CH_3COO^-< HPO_4^{2-} < SO_4^{2-} (stabilize proteins)

This rank order is also known as the "chaotropic series", as studies have shown Cl^- to have little effect on water-structure, whereas anions to the left of Cl^- are water structure-breakers (V_{disord} is dominant) called chaotropes (from the Greek, meaning disorder (*chao*)) because they destabilize proteins, while anions to the right of Cl^- are water structure-makers (V_{elect} is dominant) called kosmotropes (from the Greek, meaning order (*kosmos*)) because they stabilize proteins. Thus, polar or charged chaotropes "disrupt" the structure of water because they interact with water less strongly, while polar or charged kosmotropes interact with water more strongly than bulk water molecules with each other.

Previous work has shown that the effect of various salts on the stability of duplex DNA also follows the Hofmeister series (Hamaguchi and Geiduschek, *JACS* 84 (8),

1329-38 (1962)). In the same study it was concluded that at the very high concentrations needed to observe the anion effects, there were only minor differences observed when the cations Li^+, Na^+, K^+, and TMA^+ were varied.

The results obtained in accordance with the present invention show that the effect of anions on triplex stability follows the Hofmeister series. For the triplex $d(C^+-T)_6$:$d(A-G)_6$.$d(C-T)_6$ in 2.0 M anion at pH 7.0, rank according to triplex Tm values (°C) for the various salts is:

$NaClO_4$ (inhibits triplex formation) < NaCl (7°) < $NaOOCCH_3$ (15°); Na_2HPO_4 (15°) < Na_2SO_4 (21°) < $(NH_4)_2SO_4$ (28°).

For the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ in 2.0 M anion at pH 7.0, rank according to triplex Tm values (°C) for the various salts is:

$NaClO_4$ (44°) < NaCl (66°); $NaOOCCH_3$ (66°) < Na_2HPO_4 (80°); Na_2SO_4 (80°) < $(NH_4)_2SO_4$ (83°).

Whereas duplex DNA stability is not greatly affected by cations in general when they are at very high concentration, the applicants have found that organic cations have a strong effect on triplex stability. Their stabilizing ability can also be explained by the ion-water model. Thus, for these organic cations V_{caged} is dominant, and in this case water "structure-making" occurs as a result of the hydrophobic cation. That is, the organic cation (kosmotrope) interacts much less strongly with water, and in so doing orders the water molecules around them (the effect on the interfacial water surrounding the nonpolar substance is that it becomes more ordered).

For the triplex $d(C^+-T)_6$:$d(A-G)_6$•$d(C-T)_6$ at pH 7.0:

TPA-Cl (inhibits triplex formation) < NaCl < MA-Cl < DMA-Cl < TEA-Cl < TMA-Cl < TriMA-Cl.

For the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ at pH 7.0, the highest obtainable Tm is 72 °C in 5.0 M NaCl, while the

highest obtainable Tm is 95 °C in 6.0 M TMA-Cl.

As both triplexes and duplexes have a high negative charge density, they are stabilized in turn by cations of positive charge density. Therefore, although V_{caged} is negative for the organic cations, TMA^+ (-21 cm^3 $mole^{-1}$), TEA^+ (-18 cm^3 $mole^{-1}$), and TPA^+ (-24 cm^3 $mole^{-1}$), (because of their water structure-making nature, *i.e.*, decreased volume), TPA^+ must not have sufficient positive charge density. Thus, it is likely that the size (and hence charge density) of these organic cations also plays a role in their tendency to stabilize triplexes. At pH 7.0 they all have one positive charge and therefore their charge density will scale with their surface area (calculated using ChemPlus in HyperChem (HyperChem 4.0 (1994) Hypercube Corp., Waterloo, Ontario, Canada)): MA^+ (178 Å^2), DMA^+ (208 Å^2), $TriMA^+$ (232 Å^2) TMA^+ (252 Å^2), TEA^+ (325 Å^2), and TPA^+ (383 Å^2). This implies that $TriMA^+$ and TMA^+ have the optimum size and charge density to stabilize triplexes with homopyrimidine third strands. However, as observed, their decreasing charge density also makes them less soluble in H_2O, and this may also have an effect.

It should be noted that TEA^+ and TPA^+ have a significant destabilizing effect on the duplex $d(A\text{-}G)_6.d(C\text{-}T)_6$.

Triplexes are stabilized by certain alcohols, PEG, and DMSO as follows.

For the triplex $d(C^+\text{-}T)_6:d(A\text{-}G)_6 \bullet d(C\text{-}T)_6$ at pH 7.0:

MeOH < EtOH < 2-PrOH < 1-BuOH.

For the triplex $d(T)_{21}:d(A)_{21}.d(T)_{21}$ in 50 Vol% alcohol + MB (0.15 M Na^+/0.005 M Mg^{++}/0.01 M cacodylate titrated to the desired pH) at pH 7.0, the rank order based on Tm values (°C) is:

0%, MB only (23°) < MeOH (38°) < EtOH (53°) < 2-PrOH (65°).

For the triplex poly r(U:A•U) in Vol% EtOH + 0.016 M NaCl at pH 7.0:

0% (26°) < 10% (39°) < 20% (42°) < 30% (45°) < 50% (53°).

For the triplex $d(C^+-T)_6:d(A-G)_6 \cdot d(C-T)_6$ in 20 Vol% PEG(ave. molecular weight) + MB at pH 7.0:

0%, MB only (11°) < PEG200 (18°) < PEG400 (22°) < PEG600 (24°).

For the triplex $d(C^+-T)_6:d(A-G)_6 \cdot d(C-T)_6$ in Vol% DMSO + MB at pH 7:

0%, MB only (11°) < 10% (15°) < 20% (17°) < 40% (20°) < 50% (27°) < 60% (15°).

For the triplex $d(T)_{21}:d(A)_{21} \cdot d(T)_{21}$ in Vol% DMSO + MB at pH 7.0:

0%, MB only (23°) < 30% (34°) < 40% (38°) < 50% (15°).

Water-miscible neutral polar organic substances can also be classified as water structure-breaking (chaotropes) or water structure-making (kosmotropes) (see Collins and Washabaugh, *Q.Rev.Biophysics* (18) 323-422 (1985)). The low molecular weight alcohols are water structure-making, as is the neutral hydrophilic polymer PEG and the potent H-bond acceptor DMSO.

It would appear therefore that substances that are water structure-making enhance the stability of triplexes. Conversely, no water structure-breaking substance has been observed to enhance triplex stability. This thermodynamic model of ion-water interaction has given a thermodynamic answer. We now attempt to relate this thermodynamic understanding of how "altered water structure" may influence the conformation of DNA to the molecular mechanism for triplex formation.

The result of water-alcohol, water-PEG, or water-DMSO interaction is that it reduces the water available to hydrate other 'solutes'. This is a well known observation for DNA in water/ethanol mixtures. The higher the proportion of ethanol, the less the proportion of water available for hydration of DNA (i.e., dehydration), and in 60 to 70 % ethanol there is sufficient dehydration to induce a conformational change in DNA from B to A or Z.

Such conformational changes require varying degrees of unwinding the DNA, with resultant changes in rotation of the nucleotide residues from 36 - 45 ° to 30 - 33 ° in the case of B to A, and even *anti* to *syn* isomerization in the case of B to Z.

In this connection, the unwinding of duplex DNA increases in the presence of MeOH, EtOH, ethylene glycol and DMSO but not glycerol (Lee, et al., (1981) *Proc.Natl.Acad.Sci.U.S.A.* **78**, 2838-2842). Moreover, the degree of unwinding is a continuous process in response to the concentration of organic solvent. The Vol % of organic solvent required for unwinding increases in the order: DMSO < MeOH < EtOH < ethylene glycol.

As glycerol does not enhance triplex stability, it is likely that MeOH, EtOH, 2-PrOH, PEG and DMSO all enhance triplex stability by facilitating unwinding of the duplex. In fact, it would make sense that all compounds that facilitate both a B to A/Z transition and dehydration also enhance binding of third strands that must enter the major groove of the duplex. Clearly, third strand binding must require displacement of water from the major groove of the duplex to accommodate this extra strand. This is further supported by the observation that RecA facilitates third strand binding, since the hydrophobic environment created by the protein must facilitate removal of water (Iyer, *et al.*, *J.Biol.Chem.* 270, 14712-717 (1995)).

Thus, it appears that substances that are water structure-making enhance the stability of triplexes. They may do so by two associated mechanisms: by facilitating the unwinding of the duplex to the extent needed to accommodate the third strand, which need not necessarily involve a B to A transition, and by facilitating the removal of water from the major groove to permit third strand binding.

EXAMPLES

To illustrate the various aspects and processes of the

invention, the effects of different additives on the stability of three different triplexes is documented below. It is understood that these examples are not intended to limit the scope of the invention and that other embodiments of the invention will be apparent from the information provided to those of ordinary skill in the art.

Example 1

$d(C^+-T)_6:[d(A-G)_6 \cdot d(C-T)_6]$

$d(A-G)_6$ and $d(C-T)_6$ were synthesized, purified and analyzed as described in Lavelle and Fresco, *Nucleic Acids Res.* 23, No. 14, 2692-2705 (1995). Briefly, the strands were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 380B synthesizer. The oligomers were purified by reverse phase HPLC (0.1 M triethylammonium acetate pH 7.0/acetonitrile) and ion exchange HPLC (5 M urea/20 mM sodium phosphate pH 6.0, 5 M urea/20 mM sodium phosphate/1 M sodium sulfate pH 6.0) and desalted by reverse phase chromatography using C18 Sep-Pak. Molar extinction coefficients determined after phosphodiesterase I digestion, ε_{260} = 9890 for $d(A-G)_6$ and ε_{260} = 8510 for $d(C-T)_6$ at 25 °C in 2.6 x 10^{-5} M Tris pH 7.4 / 2.4 x 10^{-5} M $MgCl_2$, were used to determine oligomer concentration. The triplex mixture was made with equimolar stocks of the two strands; after forming the duplex, a stoichiometric amount of the third strand was added (which is the same as the homopyrimidine strand of the core duplex in this case).

Absorption spectra and thermal melting profiles were determined in a computer driven AVIV 14DS spectrophotometer equipped with a thermoelectrically controlled cell holder for cells of 1 cm pathlength. Filtered, dry air was passed through the cell compartment to prevent condensation on the cell walls at low temperatures. The flow rate was set low enough so as not to create a temperature gradient between the sample and the cell holder, which was confirmed by

monitoring the temperature in the sample and cell holder during trial melting profiles. For melting experiments, spectra were measured every 1 nm and 2 °C. Only triplex and duplex transitions that occur between 0 and 100 °C were observed. Care was taken to obtain true equilibrium melting profiles by recording scans only after a cuvette was allowed to reach the desired temperature (8 min). This ensured that the rate of temperature rise is less than the rate of the association-dissociation reaction under study, as confirmed by the absence of further absorbance change on longer incubation at some fixed temperature within the transition. These spectra were used to obtain melting profiles and their derivatives at appropriate wavelengths, from which melting transition temperatures, Tm values, were obtained from the midpoint of the transition. Tm values ($T_m \pm 0.5$ °C) were obtained by measuring each melting profile at least twice. Unless otherwise stated, Tm and % hypochromicity values were obtained from melting profiles at 260 nm. All UV-melting profiles, wavelength scans and difference spectra are plotted using raw data. % Hypochromicity was calculated using:

$$\frac{A_{260}(\text{duplex + coil}) - A_{260}(\text{triplex})}{A_{260}(\text{duplex + coil})} \times 100$$

for 3→2 + 1 transitions; and

$$\frac{A_{260}(\text{coil}) - A_{260}(\text{triplex})}{A_{260}(\text{coil})} \times 100$$

for 3→1 + 1 + 1 transitions.

The effect of various additives on triplex stability was determined, and the results are presented below in Tables 1-1 to 1-28. These results were obtained at pH 7 unless otherwise noted. The abbreviation "MB" denotes a mixing buffer comprised of 0.15 M NaCl, 0.005 M $MgCl_2$ and

0.01 M cacodylate, titrated to the desired pH.

Table 1-1--Methylammonium chloride (MA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	18	13	53	8
2.0	19	12	53	8
3.0	20	13	52	6
4.0	19	11	51	8

Table 1-2--Dimethylammonium chloride (DMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	20	11	53	8
2.0	23	10	52	8
3.0	26	9	51	6
4.0	27	10	49	8

Table 1-3--Trimethylammonium chloride (TriMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	28	10	52	6
2.0	36	9	52	7
3.0 (pH 3.7)	72(a)	19	--	--
3.0 (pH 4.9)	67(a)	15	--	--
3.0 (pH 5.8)	58(a)	15	--	--
3.0	50(a)	17	--	--
3.0 M (pH 7.4)	36	8	51	7
3.0 M (pH 7.8)	--	--	52	8
4.0 M	53(a)	16	--	--
4.0 M (pH 7.4)	36	8	50	6

(a) Tm for a 3→1 transition.

Table 1-4--Tetramethylammonium chloride (TMA-Cl)

Molarity		1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
1.0		31	10	54	9
2.0		29	9	55	7
3.0	(pH 3.7)	75(a)	18	--	--
3.0	(pH 4.9)	72(a)	16	--	--
3.0	(pH 5.8)	61(a)	14	--	--
3.0		30	10	56	9
4.0		43	9	59	7
6.0		50(a)	23	--	--
6.0	(pH 6.0)	67(a)	23	--	--

(a) Tm for a 3→1 transition.

Table 1-5--Tetraethylammonium chloride (TEA-Cl)

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.5	16	10	43	6
1.0	22	13	43	10
1.6	26	9	37	7
2.0	insoluble			

Table 1-6--Tetrapropylammonium chloride (TPA-Cl)

Molarity		1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.1		--	--	29	8
0.5		--	--	31	8
0.9		--	--	28	8
0.9	(pH 8.5)	--	--	27	8
1.0		insoluble			

Table 1-7--Cetyltrimethylammonium chloride (CTriMA-Cl)

Wt %	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	20	10
10^{-3}	--	--	23(a)	5
10^{-2}	28(b)	5	--	--
10^{-1}	insoluble, micelle formation			
10^{-4} + MB	10	11	50	13
10^{-3} + MB	22(c)	8	52	14
10^{-2} + MB	insoluble, micelle formation			
10^{-3} + MB + 0.02 M TMA	12	8	51	10
10^{-3} + MB + 0.1 M TMA	14	11	51	9
10^{-3} + MB + 0.2 M TMA	15	10	52	8
10^{-3} + MB + 0.4 M TMA	16	11	52	8
10^{-2} + MB + 0.1 M TMA	45	11	64	11
10^{-2} + MB + 0.2 M TMA	insoluble, micelle formation			

(a) Tm for duplex melting.

(b) Tm 3→2+1 transition; phase transition of CTriMA masks duplex transition.

(c) very broad transition (3-40°C).

Table 1-8--Tridodecylmethylammonium chloride (Tridodecyl MA-Cl)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	20	10
10^{-3}	--	--	20	10
10^{-3} (f)	41(d)	14	--	--
10^{-2}	insoluble, micelle formation			
10^{-4} + MB	10	10	51	11
10^{-3} + MB	11	9	51	10
10^{-2} + MB	insoluble, micelle formation			

(d) Tm for a 3→1 transition.

(f) pH 6.0.

Table 1-9--2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	21	11
10^{-4} (f)	40(d)	10	--	--
10^{-3}	22(d)	7	--	--
10^{-3} (f)	40	6	79	7
10^{-2}	27	6	77	25(e)
10^{-1}	insoluble, micelle formation			
10^{-4} + MB	10	11	50	16
10^{-3} + MB	12	7	50	15
10^{-2} + MB	insoluble, micelle formation			

(d) Tm for a 3→1 transition.

(e) significant overlap with phase transition of DOSPA.

(f) pH 6.0.

Table 1-10--Sodium Dodecylsulfate (SDS)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1 + MB	--(a)	--	51	12
1 + MB	--(a)	--	51	11
10 + MB	--(a)	--	54	12

(a) appears to inhibit triplex formation; however, any transitions below 20°C are not observable as the solution solidifies ≤20°C.

Table 1-11--Tetramethylammonium Sulfate (TMA-S)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1	16	7	48	21
0.5	20	17	56	11
1.0	25	14	57	11
1.5	TMA-S precipitates			

Table 1-12--Trehalose

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.75 + MB	12	7	47	10
1.5 + MB	12	4	44	10
2.0 + MB	13	4	41	9

Table 1-13--Glycerol

Vol %	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10 + MB	11	9	49	10
20 + MB	12	9	45	10
30 + MB	12	6	42	12
30 + MB + 1.0 M TriMA	19	8	45	9

Table 1-14--Poly(ethylene glycol) (PEG)

Vol %/(MW)	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
20 (200) + MB	18	11	44	10
40 (200) + MB	33(b)	23	--	--
20 (400) + MB	22	22	48	17
20 (600) + MB	24	15	49	14

(b) Tm for a 3→1 transition.

Table 1-15--Dimethyl Sulfoxide (DMSO)

Vol %	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10 + MB	15	12	48	12
20 + MB	17	11	45	12
40 + MB	20	12	41	12
50 + MB	27(b)	17	--	--
60 + MB	15(b)	12	--	--
60 + MB(f)	36(b)	24	--	--

(b) Tm for a 3→1 transition.

(f) pH 6.0.

Table 1-16--Mixing Buffer (0.15M NaCl; 0.005M $MgCl_2$)

pH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
4.2	32	9	62	13
5.0	29	12	50	9
7.0	11	12	50	10
7.5	1	4	50	10

Table 1-17--NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	7	7	53	12
0.5	8	14	54	10
0.8	10	16	56	11
0.9	10	17	56	11
1.0	9	17	54	12
2.0	7	13	54	12
3.0	5	2	57	7
5.0	--	--	51	10
6.0	NaCl crystallizes			

Table 1-18--Na_2HPO_4

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	8	9	54	12
0.8	12	11	56	11
2.0	15	11	57	11
2.0 (pH 6.5)	29	14	59	11
3.0	Na_2HPO_4 crystallizes			

Table 1-19--Sodium Acetate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	9	12	53	11
0.8	12	12	56	11
2.0	15	13	55	11
3.0	16	13	54	12

Table 1-20--Sodium Sulfate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	14	12	54	10
0.8 (pH 7.2)	9	8	56	11
0.8	17	13	55	12
2.0	21	14	58	11
3.0	crystallizes			

Table 1-21--Sodium Perchlorate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
2.0	--	--	42	11
2.0 (pH 6.0)	18	12	43	10

Table 1-22--Ammonium Chloride

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	8	5	56	12
0.8	--	--	54	12
2.0	--	--	58	12

Table 1-23--Ammonium Sulfate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	8	4	55	12
0.8	19	13	57	12
2.0	28	13	58	11
3.0	37(b)	14	60(b)	10

(b) overlapping transitions.

Table 1-24--Methanol (MeOH)

% MeOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10% + MB	15	13	51	11
20% + MB	15	14	48	11
30% + MB	15	12	44	12
60% + MB	16	7	37	7
70% + MB	16	7	35	7
80% + MB	no transitions observed			

Table 1-25--Ethanol (EtOH)

% EtOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10% + MB	12	12	48	10
20% + MB	13	12	44	11
30% + MB	15	6	41	11
40% + MB	15	7	36	10
50% + MB	21	23	38	8
60% + MB	40(a),(c)	31	--	--
70% + MB	no transitions observed			
50% + 1.5 M TriMA	30(c)	16	--	--

(a) broad transition (20-60°C).

(c) Tm for a 3→1 transition.

Table 1-26--2-Propanol (2-PrOH)

% Propanol	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
5% + MB	9	7	49	7
10% + MB	11	14	47	12
20% + MB	17	9	43	13
30% + MB	20	11	40	12
40% + MB	27(b)	31	39(b)	15
50% + MB	40(c)	38	--	--
60% + MB	insoluble			
30% + 20% EtOH + 3 M TMA	32(c)	10	--	--
40% + 3 M TMA	phase separation			
50% + 3 M TMA	insoluble			

(b) overlapping transitions.

(c) Tm for a 3→1 transition.

Table 1-27--1-Butanol (BuOH)

% BuOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1% + MB	8	10	51	9
1% + MB	7	9	50	9
5% + MB	7	10	47	9
10% + MB	phase separation			

Example 2

$d(T)_{21}:[d(A)_{21}\cdot d(T)_{21}]$

Triplexes were formed and tested as in Example 1, except that the strands $d(T)_{21}$ and $d(A)_{21}$ were used instead of $d(A\text{-}G)_6$ and $d(C\text{-}T)_6$. The concentrations of these strands were calculated using the molar extinction

coefficients for poly (dA)(ε_{257}=8600) and for poly (dT) (ε_{265}=8700) at 25 °C. The results are shown below in Tables 2-1 to 2-10.

Table 2-1--NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	24	17	58	19
0.8	42	18	62	17
1.0	49(a)	18	64(a)	18
2.0	66(b)	33	--	--
3.0	70(b)	33	--	--
5.0	72(b)	33	--	--
6.0	NaCl crystallizes			

(a) overlapping transitions.
(b) Tm for a 3→1 transition.

Table 2-2--Ammonium Chloride

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	65	36	--	--
2.0	71	36	--	--
3.0	76	36	--	--

Table 2-3--Ammonium Sulfate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	71	36	--	--
2.0	83	36	--	--
3.0	93	36(c)	--	--

(c) obtained by extrapolation.

Table 2-4--Trimethylammonium chloride (TriMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
1.0 + MB	45	16	61	11
2.0 + MB	66(b)	27	--	--
3.0 + MB	70(b)	26	--	--
1.0	39	17	63	18

(b) Tm for a 3→1 transition.

Table 2-5--Tetramethylammonium salts (TMA)

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
1.0 TMA-Chloride	28	17	65	19
6.0 TMA-Chloride	95(b)	33(c)	--	--
1.0 TMA-Sulfate	54	10	74	13
1.5 TMA-Sulfate	TMA-S precipitates			

(b) Tm for a 3→1 transition.

(c) obtained by extrapolation.

Table 2-6--Sodium salts (all 2.0 M)

Salt Added	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
Na_2HPO_4	80(b)	33	--	--
$NaOOCCH_3$	66(b)	33	--	--
Na_2SO_4	80(b)	33	--	--
$NaClO_4$	44(b)	30	--	--

(b) Tm for a 3→1 transition.

Table 2-7--Alcohols (all 50 Vol%)

Alcohol Added	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
Methanol + MB	38(a)	38	--	--
Ethanol + MB	53(a)	74	--	--
2-Propanol + MB	65(a)	74	--	--

(a) Tm for a 3→1 transition.

Table 2-8--Dimethyl Sulfoxide

Vol %	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
30 + MB	34(b)	18	44(b)	16
40 + MB	38(a)	35	--	--
50 + MB	15	9	34	28

(a) Tm for a 3→1 transition.

(b) overlapping transitions.

Table 2-9--Poly(ethylene glycol)

Vol % (MW)	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
20 (200) + MB	39(a)	25	--	--
40 (200) + MB	41(a)	45	--	--
20 (600) + MB	52(a)	54	--	--

(a) Tm for a 3→1 transition.

Table 2-10--0.15 M NaCl+0.005 M $MgCl_2$

	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
	23	18	53	15

Example 3

Poly(rU):Poly(rA)•Poly(rU)

Triplexes were formed using the strands Poly(rU) and Poly(rA) (the same samples used in the work of Broitman, *et al.*, (1987) *Proc.Natl.Acad.Sci. U.S.A.* 84, 5120-5124). The results in Tables 3-1 to 3-4 were obtained by the standard UV melting protocols described in Example 1.

Table 3-1--Ethanol + 0.016 M NaCl

Vol % EtOH	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10	39(a)	37	--	--
20	42(a)	39	--	--
30	45(a)	40	--	--
50	53(a)	54	--	--
60	insoluble			

(a) Tm for a 3→1 transition.

Table 3-2--Cetyltrimethylammonium chloride + 0.016M NaCl

Wt %	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10^{-4}	27	16	40	20
10^{-3}	38(b)	12	63(b)	18
10^{-2}	insoluble, micelle formation			

(b) overlapping transitions.

Table 3-3--Trimethylammonium chloride + 0.016 M NaCl

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
0.020	34	21	42	18
0.053	44(a)	37	--	--
0.600	69(a)	41	--	--

(a) Tm for a 3→1 transition.

Table 3-4--Tetramethylammonium chloride + 0.016 M NaCl

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
0.020	31	18	40	17

Table 3-5--0.16 M NaCl

1st Transition		2nd Transition	
Tm	Hypochromicity	Tm	Hypochromicity
°C	%	°C	%
26	17	40	23